Proc. Natl. Acad. Sci. USA
Vol. 84, pp. 274–278, January 1987
Medical Sciences

Effects of an extract from *Phyllanthus niruri* on hepatitis B and woodchuck hepatitis viruses: *In vitro* and *in vivo* studies

(antiviral agent/*Marmota monax*/DNA polymerase/hepatitis B surface antigen/woodchuck hepatitis surface antigen)

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Contributed by B. S. Blumberg, September 18, 1986

ABSTRACT **An aqueous extract of the plant *Phyllanthus niruri* inhibits endogenous DNA polymerase of hepatitis B virus and binds to the surface antigen of hepatitis B virus *in vitro*. The extract also inhibits woodchuck hepatitis virus (WHV) DNA polymerase and binds to the surface antigen of WHV *in vitro*. The extract, nontoxic to mice, was tested for antiviral activity in woodchucks (*Marmota monax*). In a trial using six long-term WHV-carrier woodchucks, five treated animals showed a faster decrease in woodchuck hepatitis virus surface antigen titer compared to one untreated control. In animals recently infected with WHV, the extract was effective when administered i.p. in three out of four animals in reducing and within 3–6 weeks eliminating both the surface antigen titer and DNA polymerase activity in serum. The treatment was discontinued after 10 weeks, and the treated animals have remained free of detectable markers of WHV for more than 45 weeks. In contrast, three untreated controls remained positive for both markers for WHV. One of the controls died after 8 weeks; the other two controls have remained positive for WHV markers for more than 45 weeks. In a third trial with long-term carriers, test animals treated subcutaneously with the extract for 12 weeks did not respond; but on switching the mode of administration to i.p., two out of the five animals showed a significant decrease in woodchuck hepatitis virus surface antigen titer compared to controls.**

Chronic carriers of the hepatitis B virus (HBV) may remain asymptomatic for long periods, but many are at high risk of eventually developing post-hepatitic cirrhosis and primary hepatocellular carcinoma. Carriers are often infected within the first few years of life, but symptoms of chronic liver disease and primary hepatocellular carcinoma may not be perceived until the third, fourth, or later decades; pathogenesis, even though relentless, is slow (1, 2).

Materials of animal, bacterial, and plant origin (3) have been described that appeared to interfere with the binding of the HBV surface antigen (HBsAg) to the HBsAg antibody (anti-HBs). Subsequently, about 1200 species of plant were tested, and about one-third were found to inhibit anti-HBs–HBsAg binding. To obtain more specificity and increase the probability of obtaining an effective therapeutic agent, in addition to the inhibition of HBsAg–anti-HBs binding, we examined plant extracts *in vitro* to determine if they inhibited the endogenous DNA polymerase (DNAp) of HBV, which is necessary for its replication. The first plant tested was *Phyllanthus niruri*, which has been and is used widely (4) in southern India and elsewhere for the treatment of jaundice. The treatment of HBV carriers has not been recognized in traditional indigenous medical systems. The inhibition of anti-HBs–HBsAg binding by *P. niruri in vitro* has been reported by Thyagarajan *et al.* (5).

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To assess the effects of *P. niruri* on the replication of HBV-like viruses *in vivo*, we used the woodchuck (*Marmota monax*) as an animal model. The carrier state in woodchucks and humans is similar. Liver diseases including primary hepatocellular carcinoma induced by woodchuck hepatitis virus (WHV) in woodchucks are very similar to those induced by HBV in humans. WHV is similar to HBV (6, 7) with substantial immunological cross-reactivity (8) and significant homology of DNA (9). The endogenous DNAp of both viruses exhibited optimal activities in the same range of pH, $MgCl_2$ concentration, and showed similar sensitivity to inhibitors like phosphonoformic acid and arabinofuranosyl nucleotides (10).

In this paper we report that *P. niruri* has profound effects *in vitro* on HBsAg, on woodchuck hepatitis virus surface antigen (WHsAg), and on the DNAp of both viruses and *in vivo* on the replication of WHV and on liver histopathology. In some controlled studies, it appeared to eliminate WHV from carriers.

MATERIALS AND METHODS

Preparation of the Aqueous Extract of *P. niruri*. Dried whole plant (40 g) was pulverized in a Waring blender and mixed with 200 ml of water. The mixture was shaken periodically (60°C) for 2 hr and filtered through nylon mesh. The filtrate was centrifuged at 8000 rpm for 1 hr in a Beckman JA10 rotor at 20°C. The supernatant was filtered through a 0.45-μm filter (Millipore) for *in vivo* studies.

Assay for HBsAg or WHsAg Binding Activity. Serial dilutions of the aqueous extract of *P. niruri* were mixed with an equal volume of sera positive for HBsAg or WHsAg, and the mixture was incubated for 1 hr at 20°C. The mixture was assayed directly for HBsAg or cross-reacting WHsAg using Ausria II ELISA kits (Abbott). Binding activity was expressed as the decrease (in percent) in the absorption of the test sample compared to that of the control composed of 1:1 (vol/vol) mixture of surface antigen positive serum and PBS. (PBS = 0.01 M sodium phosphate/0.85% NaCl, pH 7.2.)

Assay of WHsAg Titers in Serum. Serum titers of WHsAg were determined by assaying serial dilutions of serum with the Ausria II kit using the value obtained for sera of uninfected woodchucks as controls.

Inhibition of Endogenous Viral DNA Polymerase Activity. Suspensions of the virus were added to a reaction mixture containing the nucleotides required for DNA synthesis by DNAp. The formation of DNA was determined by gel electrophoresis. Serial dilutions of the extracts of the plant were added to determine their inhibitory ability.

Abbreviations: DNAp, DNA polymerase; HBV, hepatitis B virus; WHV, woodchuck hepatitis virus; WHsAg, woodchuck hepatitis surface antigen; HBsAg, hepatitis B surface antigen; anti-HBs, antibody to HBsAg.

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Table 1. Effect of *P. niruri* extract on the binding of HBs to HBsAg or WHsAg

P. niruri extract, mg/ml	% inhibition of anti-HBs binding	
	WHsAg	HBsAg
5	61	63
2.5	45	35
1.25	25	18
0.63	11	9
0.5	13	0
0.31	13	0
0.1	5	0

Serum (50 μl) containing HBV (or WHV) particles was layered over a 10–20% sucrose gradient and centrifuged at 45,000 rpm for 3 hr in a SW 55 Ti rotor in a Beckman ultracentrifuge. Pelleted virus was separated from the supernatant and was resuspended in 5 μl of 0.05 M Tris·HCl, pH 8.0. Equal volume of serial dilutions of the extract of *P. niruri* was mixed with the virus suspension. Then nucleotide triphosphates dATP, dGTP, dCTP, and [^{32}P]dTTP were added in the presence of 0.05 M Tris·HCl, pH 8.0, containing 10 μM $MgCl_2$, 0.15 M NaCl, 1 mM dithiothreitol, and 0.1% Nonidet P-40, and the mixture was incubated at 37°C for 2 hr. For control, the virus suspension was mixed with 0.05 M Tris·HCl buffer, pH 8.0, instead of the potential inhibitor. The reaction was stopped by the addition of 15 μl of Pronase (0.5 mg/ml) in 0.1% $NaDodSO_4$ containing 0.01 M EDTA in Tris·HCl, pH 7.4; and the mixture was electrophoresed on 1.5% agarose gel using bromophenol blue as the tracking dye. The DNA formed was detected by autoradiography.

A quantitative determination of the inhibition was obtained by substituting [^{3}H]dTTP and [^{3}H]dGTP for [^{32}P]dTTP as radiolabel according to the method reported by Hantz *et al.* (10) with modifications. In this experiment the reaction was terminated by the addition of 2.5 ml of 5% (wt/vol) trichloroacetic acid [containing 2% (wt/vol) pyrophosphate]. Then 40 μl of 2.5% bovine serum albumin and 100 μl of 5% (wt/vol) calf thymus DNA were added as carriers, and the mixture was filtered through a glass fiber filter (Whatman). The filter was washed thrice with 5% (wt/vol) trichloroacetic acid containing pyrophosphate, thrice with 95% (vol/vol) ethanol, dried under a heat lamp, and the radioactivity remaining on the filter was measured in a scintillation counter (Packard Instrument, Downers Grove, IL).

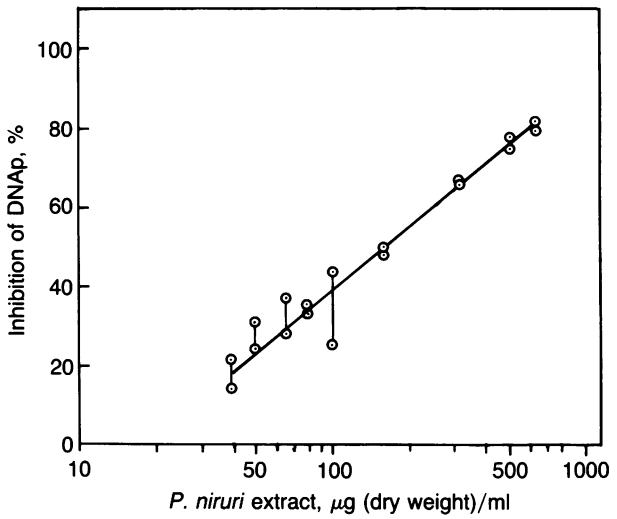

FIG. 1. Inhibition of DNAp of WHV by aqueous extracts of *P. niruri*. The data are presented as μg (dry weight) per ml of assay mixture.

To determine the WHV DNAp activity in sera of woodchucks used for experiments *in vivo*, a virus pellet, centrifuged from 50 μl of serum on sucrose density gradient, was used directly without addition of any diluent buffer as described above.

RESULTS

The Effect of *P. niruri* Extract on WHsAg and HBsAg *in Vitro*. *P. niruri* extract inhibits the reaction of HBsAg with anti-HBs and of WHsAg with anti-HBs (Table 1). The inhibition was concentration dependent for HBsAg and WHsAg.

Inhibition of WHV DNAp by the Extract *in Vitro*. Serial dilutions were assayed for their ability to inhibit WHV DNAp activity. Inhibition (Fig. 1) was directly proportional to the concentration of the extract up to 600 μg/ml at which the inhibition was 82%.

Toxicity Tests in Mice. Forty outbred albino female mice were used to determine the toxicity of the extract in accordance with a National Institutes of Health recommended assay to determine acute toxicity. The mice were divided into eight

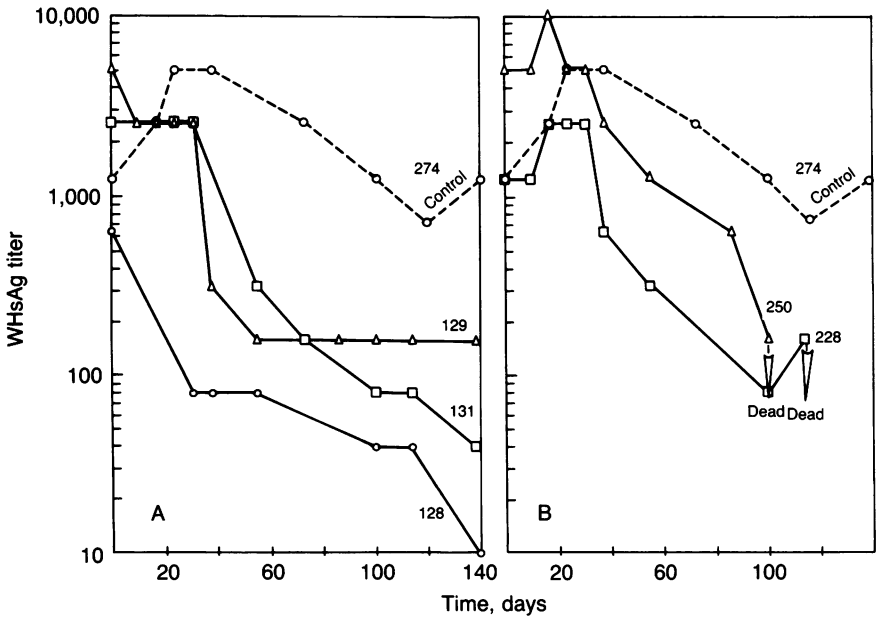

FIG. 2. Effect of aqueous extracts of *P. niruri* given i.p. on long-term chronic-carrier woodchucks (solid lines) compared to a single control carrier (dashed lines). For clarity, the results have been arbitrarily separated in two groups, *A* and *B*. The numbers of the animals are given next to the appropriate curve.

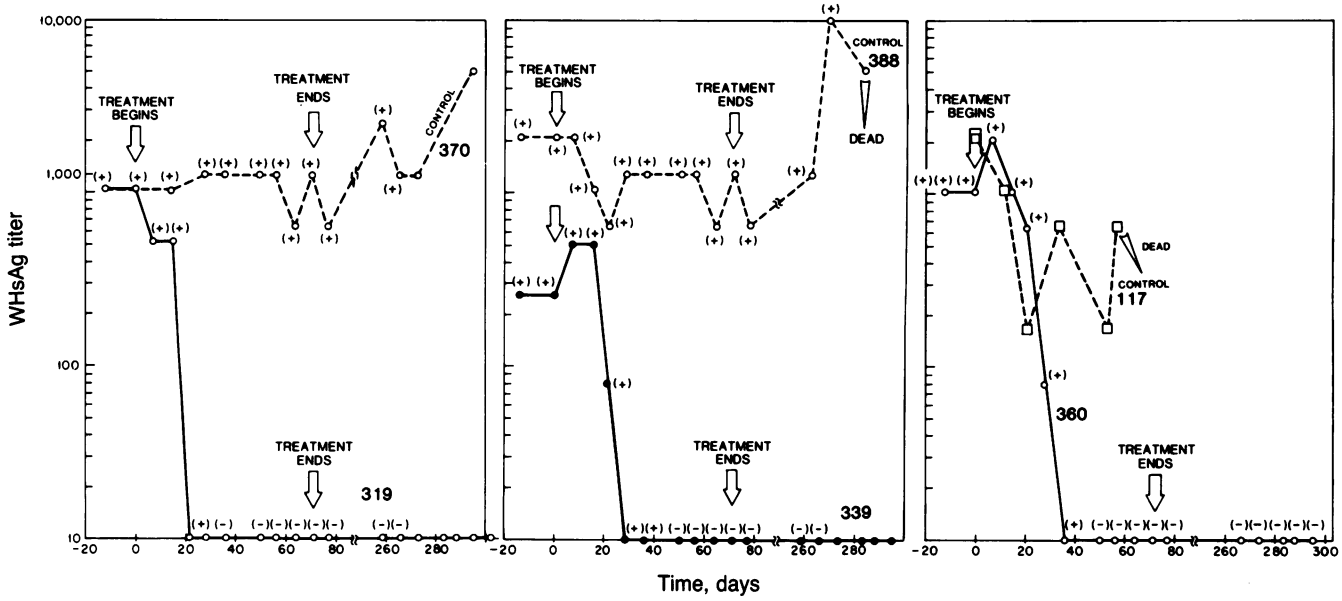

FIG. 3. Effect of aqueous extracts of *P. niruri* given i.p. on recently infected woodchucks. Solid lines indicate treated animals, and dashed lines indicate the controls. Arrows indicate the beginning and end of treatment. The DNAp activity [positive (+) or negative (−)] is indicated in parentheses.

sets of five mice and were weighed. Four sets designated "test" were given i.p. aqueous extract of *P. niruri* [0.1 ml, 1.8 mg (dry weight) per mouse], while the other four sets, designated "control," were given i.p. PBS, 0.1 ml per mouse. All sets were weighed after 3 and 7 days. There was no loss in weight among the treated or control mice after 3 days. There was a net gain in weight among all the mice after 7 days. One of the treated mice died on the 3rd day of internal injury unrelated to the test.

Long-Term Chronic-Carrier Woodchucks. An initial study was conducted on woodchuck carriers whose sera showed the presence of both the surface antigen and WHV DNAp activity at the time these animals were trapped. The length of time these animals were carriers of WHV was unknown, but it was probably more than several months. Of the six carrier woodchucks that were available, five animals, 128, 129, 131, 228, and 250, were treated; and one, 274, was used as control. Test animals were given 0.5 ml of the extract [9 mg (dry weight)] i.p. once a week, while the control animal was given the same volume of buffered saline i.p. once a week. The animals were bled periodically, and the titer of WHsAg was determined (Fig. 2). A comparison of the slope of the curves indicated that the extract appeared to reduce the titer of WHsAg in long-term chronic carriers.

Woodchucks Recently Infected with WHV. The second study was conducted on animals that were negative for WHV at the time of capture but became positive while in the enclosures in our woodchuck colony. By the time of the experiment, these animals had been infected for at least 1 month. The aqueous extract of *P. niruri* [0.5 ml, 9 mg (dry weight) per woodchuck] was administered i.p. twice a week to four woodchucks, 319, 339, 360, and 376A; the other three, 370, 388, and 117, received 0.5 ml of PBS twice a week. They were followed by weekly bleeding and assay for WHsAg and WHV DNAp. Treatment was terminated after 72 days, but the weekly bleedings were continued for over 300 days. Liver biopsy was performed on day 80, except for control woodchuck 117, which was autopsied when it died on day 57.

In woodchuck 319, WHsAg started dropping soon after the start of treatment, becoming undetectable about 21 days later (Fig. 3). WHV DNAp activity stayed positive for about 1 week after the surface antigen titer became undetectable, but subsequently it also became undetectable. Although the treatment with extract was terminated after 72 days, there were no detectable levels of the surface antigen or DNAp

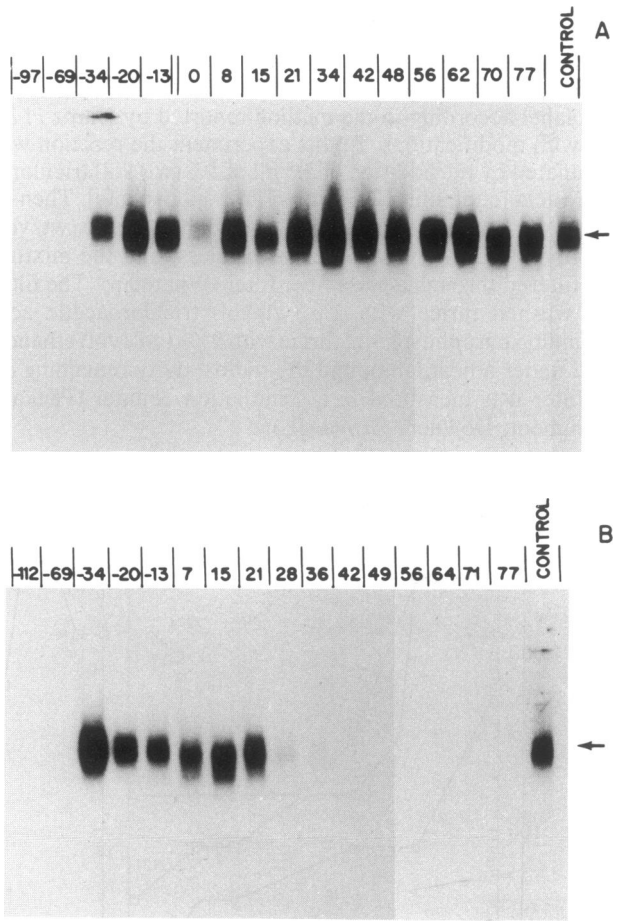

FIG. 4. Autoradiography of DNAp activity on the day indicated (−, before treatment) in the sera of a control animal, 370 (*A*) and a treated animal, 339 (*B*). These are typical of the other two control and treated animals (see Fig. 3). Arrow indicates the band of 3.3-kilobase, closed, circular DNA of WHV.

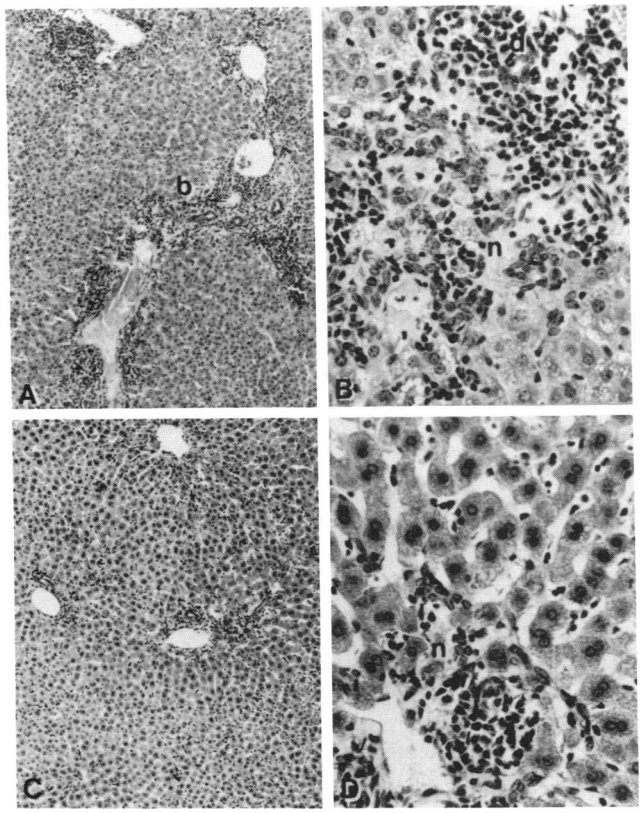

FIG. 5. Comparison of livers from the untreated woodchuck 370 (*A* and *B*) and the treated woodchuck 339 (*C* and *D*) that were infected with WHV and obtained at biopsy 8 days after the termination of treatment. All preparations were sectioned in paraffin at 5 μm and stained with hematoxylin and eosin. (*A*) Typical pattern of chronic progressive viral hepatitis with granulomatous inflammation centered chiefly around portal triads with frequent "bridging" intervening spaces between them (b). Note blurring of intralobular cords as compared with *C* (treated), owing to inflammatory swelling of individual hepatocytes. (×75.) (*B*) A periportal lesion, with an agglomeration of lymphocytes, plasmacytes, histiocytes, fibroblasts, and necrotic hepatocytes (n); proliferating biliary ductules (d) are conspicuous, which, along with fibrosis, indicate a progression to cirrhosis. (×300.) (*C*) Minimal inflammatory foci are barely visible in the portal triads of this treated animal. Hepatocytic swelling and loss of crispness of cords is seen to the right of center. (×75.) Overall, these effects are much less than in the untreated liver. (*D*) Small periportal granuloma; hepatocyte and cords are well preserved, apart from rare necrosis (n). (×300.)

activity in 319 up to 300 days after the start of treatment (i.e., 228 days after termination of treatment). The control animal 370, on the other hand, did not show a drop in either WHsAg titer or DNAp activity up to 300 days.

Treated animals 339 and 360 showed a similar drop in WHsAg between 21 and 35 days after the start of treatment, followed 7–14 days later by a drop in WHV DNAp; the markers stayed undetectable up to 300 days. Control animals 117 and 388, however, showed high levels of the WHsAg and DNAp during the same period. Autoradiography of the product of DNAp reaction in the serial bleedings of control woodchuck 370 and *P. niruri* extract-treated woodchuck 339 is given in Fig. 4. The presence of a band at 3.3 kilobases (arrow) indicated WHV DNAp activity up to 21 and 28 days. These became undetectable thereafter. DNAp of control animal 370 did not change.

Woodchuck 376A, one of the four treated animals, became bacteremic early in the experiment. Chloramphenicol was administered, but the animal succumbed to the infection. It did not respond to the extract.

The histopathology of the livers of control woodchuck 370 and treated animal 339 are shown in Fig. 5. Data on the liver biopsy performed 8 days after the termination of treatment are given in Table 2. (Liver biopsies before treatment were not available.) The three untreated controls, 370, 388, and 117, showed extensive portal infiltration and focal necrosis; all three were diagnosed as chronic active hepatitis. The livers of the treated animals 319, 339, and 360, on the other hand, showed marginal or negative portal infiltration and focal necrosis. The diagnosis of woodchuck 319 was early mild viral hepatitis, of 339 was early active hepatitis, and of 360 was minimal portal hepatitis.

Table 2. Data on liver biopsies performed 8 days after termination of treatment in experiment involving woodchucks recently infected with WHV

	Pathology		
	Portal infiltrate	Focal necrosis	Diagnosis
Control animal			
370	+++	++	Chronic, active hepatitis
388	++	++	Chronic, active hepatitis
117	+++	++	Chronic, active hepatitis
P. niruri extract-treated animal			
319	±	±	Mild viral hepatitis
339	+	+	Active hepatitis
360	±	0	Minimal, portal hepatitis

0, None. ±, Marginal. +, Minimal positive. ++, Positive. +++, Extensive.

Subcutaneous Administration of the Extract in Long-Term Carrier Woodchucks. Five of eight long-term WHV-carrier woodchucks (327, 437, 456, 488, and 492) were administered 0.5 ml [9 mg (dry weight)] of extract subcutaneously twice a week. The remaining three (318, 429, and 471) were given 0.5 ml of PBS subcutaneously twice a week. The animals were bled weekly, and the titer of WHsAg and WHV DNAp was monitored. During 3 months of treatment, there was no appreciable change in either marker in the treated or control animals.

We concluded that subcutaneous administration was ineffective. We hypothesized that either the active principle was not absorbed by this route, that antibodies were developed against the extract, or some other mechanism rendered it ineffective. After 90 days the mode of administration was changed to the intraperitoneal route that had apparently been successful in the previous studies. Two of the treated animals showed a drop in WHsAg about 60 days after switching to i.p. administration (Fig. 6). One control and two treated animals died due to bacteremic infections. None of the control animals showed any significant change in WHsAg.

DISCUSSION

London and Blumberg (1) have proposed a model to explain the observations on the relation between primary hepatocellular carcinoma and HBV. It postulates the existence of fully differentiated liver cells which, when infected, allow complete replication of HBV. (They are designated S cells; i.e., susceptible to replication.) Less-differentiated liver cells (common in the fetus and newborn but less so in the adult liver), when infected, do not allow replication, although penetration of the virus and integration of virus DNA may occur. (They are designated R cells; i.e., resistant to repli-

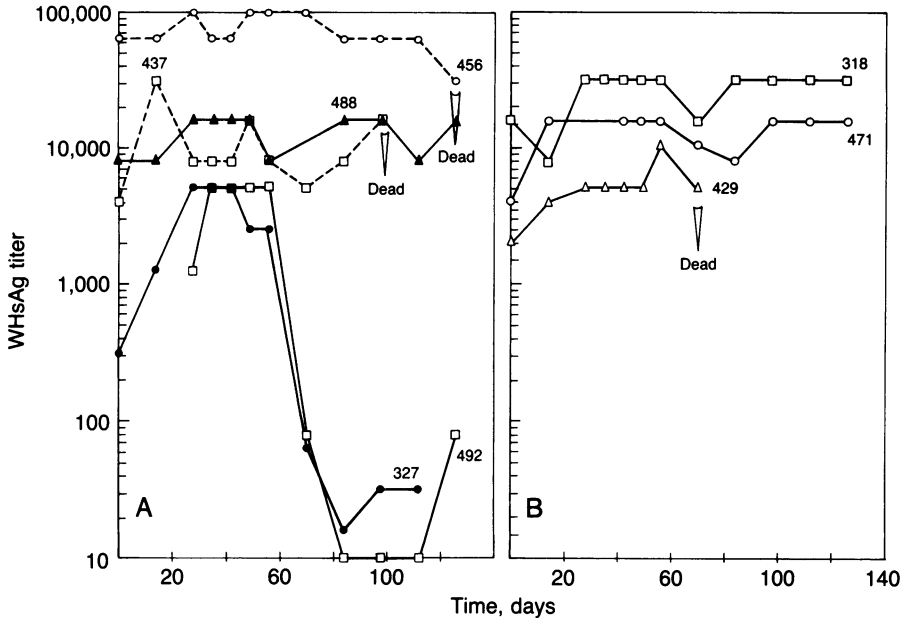

FIG. 6. Effects of *P. niruri* extracts given i.p. to animals that did not respond to subcutaneous administration of the same extract. (*A*) Treated animals; (*B*) controls.

cation.) The proliferating HBV in the differentiated cells leads to cell death, in part caused by the immune response of the host to its S cells altered by HBV. The less-differentiated R cells are not damaged by the virus, since the virus does not replicate. The R cells divide and multiply in response to the death of the S cells. With increased division of R cells, chromosomes are more liable to disruption, deletion, rearrangement, or mutation by the resident HBV (or, possibly, another agent). This could result in a favored clone that divides rapidly and is eventually perceived as a cancer.

The course of these events could be stopped if virus were eliminated from the carrier. If this is not possible, then decreasing the virus load or inhibiting its entry into liver cells could slow the death of liver cells so that perceptible disease would not be expected to occur until the carrier had lived out a life span. We have referred to this as "prevention by delay" (2).

In an attempt to achieve this goal, we looked for agents that would affect the virus or its entry into liver cells. The aqueous extract of *P. niruri* inhibits HBV DNAp and WHV DNAp and interferes with the binding of anti-HBs to HBsAg and WHsAg apparently because of its ability to bind the surface antigen.

The extract of *P. niruri* was tested in three independent *in vivo* experiments in woodchuck carriers. In the first, with long-term carriers, a significant drop in the titer of WHsAg was observed in the extract-treated animals compared to the control. In the second, using short-term carriers, the WHsAg and WHV DNAp in three of four treated animals became undetectable and stayed that way even after the treatment was terminated, while the levels of these markers stayed high in the controls. These results indicated a possible break in the carrier state directly attributable to the treatment with *P. niruri* extract. The conclusion from the third experiment was that the extract was not effective when administered subcutaneously. However, on switching the mode of administration to i.p., two out of three extract-treated animals showed a drop in WHsAg titer. The advantages of intraperitoneal chemotherapy for liver ailments have been reported (11) and may be applicable to WHV and HBV infections since replication of both these viruses takes place in the liver. Although this experiment was faulted because of its *post hoc* design, the results were in the direction predicted from the two earlier experiments.

Our preliminary results indicate that there are one or more active materials in *P. niruri* that inhibit the replication of WHV *in vivo* and decrease the pathological effects of WHV on woodchuck liver. By inference, the substance should affect HBV infection in humans similarly. Using a variety of techniques (including HPLC), we have identified fractions from *P. niruri* containing the DNAp inhibitory activity and the surface antigen binding activity.

We are indebted to Philip Custer for the histopathological studies, Howard Blatt for the biopsies, and Terry Halbherr and Renee Grob for technical assistance. The research was supported in part by grants from the Kaiser Family Foundation, the Mary L. Smith Charitable Lead Trust, and the Commonwealth of Pennsylvania.

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